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Study of Reproductive Activity, Maturity Stages and Tetrodotoxin (TTX) in Yellow Pufferfish, *Xenopterus naritus*

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Bachelor of Science with Honours
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This dissertation is submitted in partial fulfillment of the requirement for the degree of Bachelor Science with Honours in Aquatic Resources Science and Management

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DECLARATION OF AUTHORSHIP

I, Herman Hermawan Bin Bakri (41412) declare that the final year project report entitled:

Study of Reproductive Activity, Maturity Stages and Tetrodotoxin (TTX) in Yellow Pufferfish, *Xenopterus naritus*

and the works presented in the report are both my own, and have been generated by me as the result of my own original research. I confirm that:

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List of Abbreviations

cm	Centimetres
FSTS	Fakulti Sains dan Teknologi Sumber
GPS	Global Positioning System
GSI	Gonadosomatic Index
HSI	Hepatosomatic Index
H&E	Haemotoxylin & Eosin
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
MU/g	Mouse Unit per gram
STX	Saxitoxin
TTX	Tetrodotoxin
UNIMAS	Universiti Malaysia Sarawak

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ABSTRACT

This study was carried out in order to document the reproductive activity, maturity stages and tetrodotoxin (TTX) distribution in yellow pufferfish, *Xenopterus naritus* from Batang Saribas, Sarawak. The reproductive activity study shows the result of gonadosomatic index (GSI), hepatosomatic index (HSI) and fecundity, which both male and female individuals are found to be the highest in August (GSI: male-0.95%, female-12.73%; HSI: male-3.44%, female-4.04%; mean fecundity-8304.17), followed by September and October 2015. Maturity stage determination shows that August individuals are classified under late stages of seven-stage maturity scale, while September and October individuals are classified under early stages of the scale. Liver condition of this species also was observed and variations in terms of size, weight and color of liver are obtained. Besides, the presence and level of tetrodotoxin (TTX) in four body parts, which are muscle (72.3 MU/g), gonad (167.4 MU/g), liver (184.9 MU/g) and intestine (185.1 MU/g) were determined by using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS).

Keywords: *Xenopterus naritus*, maturity stage, tetrodotoxin, histology, chromatography

ABSTRAK

Kajian ini dijalankan untuk mendokumenkan aktiviti reproduksi, tahap kematangan dan taburan tetrodotoksin (TTX) dalam ikan buntal kuning, Xenopterus naritus *dari Batang Saribas, Sarawak. Kajian aktiviti reproduksi menunjukkan keputusan analisa indeks gonadosomatik, indeks hepatosomatik dan fekunditi individu jantan dan betina yang mencatatkan bacaan tertinggi pada bulan Ogos (GSI: jantan-0.95%, betina-12.73%; HSI: jantan-3.44%, betina-4.04%; min fekunditi-8304.17) diikuti bulan September dan Oktober 2015. Penentuan tahap kematangan menunjukkan individu dari bulan Ogos dikelaskan dalam tahap lewat pada skala kematangan tujuh tahap, manakala individu dari bulan September dan Oktober dalam tahap awal pada skala tersebut. Keadaan hati spesis ini juga diperhatikan dan kepelbagaian dari segi saiz, berat dan warna hati telah didapati. Selain itu, kehadiran dan tahap tetrodotoksin (TTX) dalam empat bahagian badan, iaitu, otot (72.3 MU/g), gonad (167.4 MU/g), hati (184.9 MU/g) dan usus (185.1 MU/g) telah ditentukan menggunakan kromatografi cecair-spektometri jisim tandem (LC-MS/MS).*

Kata kunci: Xenopterus naritus, *tahap kematangan, tetrodotoksin, histologi, kromatografi*

1.0 Introduction

Yellow pufferfish, *Xenopterus naritus* is a well-known pufferfish species amongst the local people in Sarawak. It is a migratory species that inhabits the South China Sea and returns to the river to spawn. *X. naritus* is widely distributed in China, Thailand, Vietnam, Indonesia and Malaysia. It is a brackish species, in which can be found at the coastal areas as well as along the river, ranging from the estuary until the middle stretch of the river, or when the water salinity reaches zero (Gambang and Lim, 2004).

In Malaysia, *X. naritus* is abundant only in Sarawak. This fish can be easily found in the coastal waters especially in areas fringing the mangroves along Batang Saribas in Betong, Sarawak. This species can be recognized by its physical appearances, which are having green-brownish upper body part and a prominent yellow at lower body part. Locally known as 'ikan buntal kuning', it is considered a delicacy by the local community particularly in the Kampung Manggut area. Due to its uniqueness, it has become a tourist attraction in Sarawak through the 'yellow puffer fish festival' celebrated every year in August at Kampung Manggut, Betong, Sarawak (Azman *et al.,* 2013).

Most of the pufferfish in Family Tetraodontidae, including *X. naritus* contain neurotoxins especially Tetrodotoxin (TTX). According to previous study done by Noguchi *et al.* (2006), TTX can be found accumulated in various body parts of this species, which are skin, muscle, liver, intestine and gonad. Noguchi and Ebesu (2001) in another study revealed that the toxicity in TTX is very dangerous and toxic as it can causes the death to human, considered that pufferfish as the second most poisonous vertebrates in the world.

Although the existence of dangerous toxin in *X. naritus* is already detected and confirmed by previous studies, local people in Sarawak still consume this fish as their regular diet. They usually consume this fish for delicacy despite the tetrodotoxin (TTX) content in its body (Azman *et al.*, 2013). This is because most of the local people usually have a remarkable skill on removing the internal organs of the fish before preparing it as delicacies.

Study of pufferfish in general has been done in various places previously, involving different parameters and focus. However, a detailed study of biology and population dynamics by using the integrated study which comprises of reproductive activity and maturity stages of *X. naritus* has not been done or attempted in Sarawak. Besides, there is still no documented report about it until now. Therefore, this project was focused on the study of reproductive activity, maturity stages and TTX in *X. naritus*. The findings of this study are expected to be useful for the conservation effort of *X. naritus* in Malaysia.

Therefore, the objectives of this study are to:

1) Determine the mean GSI, HSI and fecundity in *X. naritus* in from August to October 2015.
2) Determine the maturity stages of *X. naritus* from August to October 2015.
3) Determine the presence and level of TTX in *X. naritus* in different body parts.

2.0 Literature Review

2.1 Pufferfish

In general, pufferfish has various genera and species, including *Lagocephalus sp.*, *Takifugu sp.* and *Sphoeroides sp.* They are all known to have toxin such as TTX and STX in their body (Ngy *et al.*, 2009) and being distributed in different parts of their body (Noguchi *et al.*, 2006). Despite for having such dangerous toxin, people around the world still take pufferfish as their consumption.

For example, in Malaysia, local people of Sarawak, especially in Kampung Manggut, Betong consume yellow pufferfish as delicacy. In Japan, people consume tiger blowfish, or 'torafugu' as special dish in many restaurants. Besides, in Mexico, local people already consider pufferfish species from their coastal waters as non-toxic and safe for consumption. Thus, Mexico has become main exporter of pufferfish product in the world (Vasquez *et al.*, 2000).

Pufferfish has known for various local names. It is also called as blowfish, globe fish, toad fish or balloon fish (Gambang and Lim, 2004) by many people from different geographical region. This fish belongs to Family Tetraodontidae. Etymologically, the word tetraodontidae is derived from Greek word, means 'four-teeth' (Kalogirou, 2012). This suits the morphology of pufferfish as they are having two pairs of teeth, making them easier to crush their natural prey, which are mollusk and crustacean.

In ecosystem, pufferfish maintains their population by having a special self-defense mechanism in order to protect themselves against the threat of predators. This includes their unique ability to inflate their body by swallowing water or air (Kalogirou, 2012), as shown in Figure 1. This will prevent the predator to eat them and will scare away the predator, as they seem bigger than the predators. This mechanism also initiated when they feel stressed or threatened. Apart from this, in previous study carried out by Itoi *et al.* (2014), discovered that larvae of *Takifugu sp.* also protect themselves by using a low concentration of maternal TTX in order to prevent predators from swallowing them.

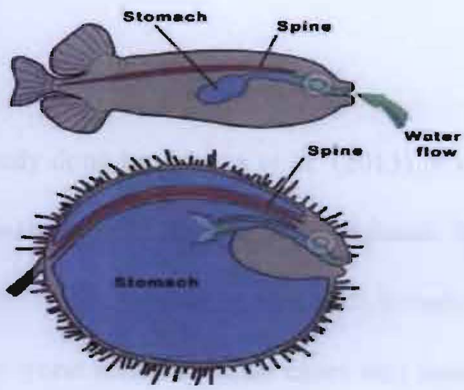

Figure 1.Inflatation mechanism of pufferfish

2.2 Yellow Pufferfish

Yellow pufferfish, *Xenopterus naritus* can be easily recognized by its green-brownish upper body part and a prominent yellow coloration at lower body part, as shown in Figure 2. Compared to other species of pufferfish, *X. naritus* has a more rigid abdominal structure. Thus, it makes this species more difficult to inflate themselves, compared to other pufferfish (Chulanetra *et al.*, 2011).

However, it still has spines on its body, which can be used to defend themselves against predators.

X. naritus is a well-known pufferfish species amongst the local people in Sarawak. It is a migratory species that inhabits the South China Sea and returns to the river to spawn. Therefore, it is a brackish fish and in order to keep it in captivity for this project, clean seawater must be used in their aquarium. It is widely distributed in China, Thailand, Vietnam, Indonesia and Malaysia. In Malaysia, it is abundant only in Sarawak and people usually consume this fish for delicacy despite the tetrodotoxin content in its body (Azman *et al.*, 2013).

According to previous study done by Azman *et al.* (2013), it is confirmed that *X. naritus* possessed a significant amount of TTX, in which can cause food poisoning if consumed without proper preparation, which requires special skill to remove the internal organs such as intestines and liver. In worst scenario, lethal cases may happen. This includes the few cases that happened in Malaysia, Thailand and Mexico (Vasquez *et al.*, 2000).

X. naritus have their own annual breeding season, which are from April until November (Gambang and Lim, 2004), in which they return to river to spawn. Therefore, in order to catch this species, it must be done within this period.

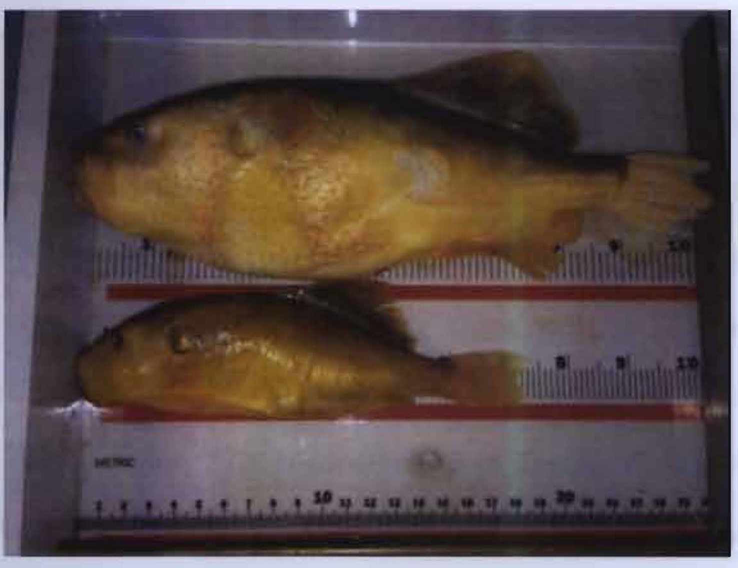

Figure 2.Yellow pufferfish, *Xenopterus naritus* (above: female, below: male)

2.3 Tetrodotoxin (TTX)

According to Ito *et al.* (2006), Tetrodotoxin (TTX) is one of the most potent non-peptide neurotoxin as it has a unique chemical structure and it is able to block sodium channels of excitable membranes in nervous system. Thus, it can lead to paralysis and eventually, death. It is also recognized as one of the strongest marine paralytic toxins made by non-protein organic compound known as aminoperhydroquinazoline (Sabrah *et al.*, 2006). Its name is derived from the name of Family Tetraodontidae (Sabrah *et al.*, 2006) as this neurotoxin can be found in species under Family Tetraodontidae including balloon fish, porcupine fish and pufferfish (Bayhan and Kaya, 2015). The chemical structure of TTX is shown in Figure 3.

In addition, TTX also can be found in other animals such as gobies, blue-ringed octopuses, carnivorous gastropods, starfish, toxic crabs, horseshoe crabs, flat worms and ribbon worms (Miyazawa and Noguchi, 2001). This shows that TTX is not unique to *X. naritus* only. This is because the TTX is not produced naturally in their body. Instead, it is produced by other marine bacteria which are *Vibrio sp.* and *Shewanella sp.* and it eventually accumulated in all these animals, including *X. naritus* through food chain (Itoi *et al.*, 2012). In *X. naritus*, TTX is concentrated in liver, ovaries, intestines and skin (Bayhan and Kaya, 2015; Tatsuno *et al.*, 2013). Besides, it is also contained in their skin and mucus (Ito *et al.*, 2006; Tatsuno *et al.*, 2013).

In other study done by Ikeda *et al.* (2010), it is revealed that high concentration of TTX is found in ovary and liver, while low concentration of TTX is found in muscle and skin of pufferfish. This shows that female pufferfish are having higher and stronger toxicity than male pufferfish. This statement also supported by study done by Itoi *et al.* (2012) which discover that the amount of TTX in pufferfish is sex-dependent. Female pufferfish also is known to transfer TTX into their larvae by accumulating the toxin in their ovaries before passing it to their eggs (Itoi *et al.*, 2014). In pufferfish larvae, the TTX is found to be localized on their body surface (Itoi *et al.*, 2014). TTX in the pufferfish larvae is mainly for self-defense purpose against predation. According to Itoi *et al.* (2014), even though the amount of TTX in pufferfish larvae is very small and does not have lethal effect on the predator, it is still enough to prevent predator from swallowing them.

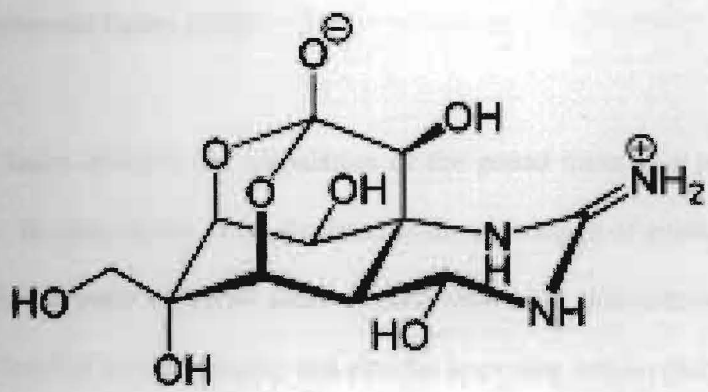

Figure 3. Chemical structure of TTX

2.4 Poisoning Case Due to Consumption of Pufferfish

So far, there are only two cases of mortality due to the consumption of this species has been reported, which is related with the consumption of dried salted yellow pufferfish eggs, in Saratok, Betong, Sarawak (Azman *et al.,* 2013). Although puffer fish consumption is rare in other parts of Malaysia, cases of food poisoning from this fish have been reported to occur.

Besides that, food poisoning also has been reported from different geographical regions due to ingestion of puffer fish and the lethality depending on the concentration of TTX present in the consumed fish tissues (Chou *et al.,* 1994). For example, the selling of all products of pufferfish, including *X. naritus*, has already banned in Thailand, as they are a significant number of fatal cases related to pufferfish happened in that country (Chulanetra *et al.,* 2011). Besides, at least 18 poisoning cases related to pufferfish consumption also officially reported in Baja California peninsula, Mexico (Vasquez *et al.,* 2000).

2.5 Gonadosomatic Index (GSI)

Gonadosomatic Index (GSI) is the calculation of the gonad mass as a proportion of the total body mass. In other words, it is calculated as the percentage of gonad mass over total body mass. It is the ratio of gonad mass to body mass and this ratio is often used for determining the level of sexual maturity and also the spawning season (Sabrah *et al.*, 2006) for any species, including *X. naritus*. Higher value of GSI indicates the higher level of maturity. Besides, a relatively high value of GSI at a particular time reveals that it is the spawning season of the Yellow pufferfish.

However, the environmental condition of habitat also plays a role in influencing the GSI level of *X. naritus*. According to Hassanin *et al.* (2002), it is discovered that waste from sewage, industrial effluent, and agricultural runoff may serve as estrogenic chemicals and will affect the reproductive functions of many vertebrates. In the case of *X. naritus* from Betong, Sarawak, the GSI level also may be influenced by domestic waste from the nearby village settlements.

In addition, the presence of such contamination can also lead to the alteration of testis morphology in male individuals, causing the reduction in spermatogenesis or sperm production (Jobling *et al.*, 1995). This in turn will cause a decrease in offspring production of the species.

Based on another study of carps done by Gimeno *et al.* (1998), exposure on the same substances in long term, which is in different life stages, will cause induced hermaphroditism. In this occurrence, the carps will undergo sequential hermaphroditism, in which fish that are born male, will change sex to female, or termed as feminization. This also will greatly reduce the number of eggs fertilization, and less offspring will be produced.

The analysis of GSI can also be used to determine the maturity stages of *X. naritus*. Habib (1979) has formulated a series of seven-stage maturity scale to describe maturity level of pufferfish, *Uranostoma richei* from Lyttelton Harbor, New Zealand. In this study, maturity stage of *X. naritus* was also described by using the scale as reference. However, the scale was modified in order to suit this species.

2.6 Hepatosomatic Index (HSI)

Hepatosomatic Index (HSI) is the calculation of the liver mass as a proportion of the total body mass. In other words, it is calculated as the percentage of liver mass over total body mass. It is the ratio of liver mass to body mass and this ratio is often used for measuring the health condition (Sadekarpawar and Parikh, 2013) of any species, including *X. naritus*.

Besides that, this ratio also can be used as biomarker to measure pollution level and presence of environmental stressors in a particular habitat (Sadekarpawar and Parikh, 2013).

Higher value of HSI shows a healthier species while lower value of HSI shows an unhealthy species. A low value of HSI also indicates that *X. naritus* are experiencing environmental stress and their habitat is badly polluted. As liver is a metabolic organ, it carries out metabolic reaction and it works best when the environmental condition is favorable for them, resulting in a bigger and heavier liver. This in turn will cause the HSI value become higher (Sadekarpawar and Parikh, 2013).

Apart from this, liver also serve as the organ of detoxification. In order to detoxify excessive toxic and pollutants, the tissue of the liver was damaged and losing mass. This will result in a lower value of HSI (Sadekarpawar and Parikh, 2013). Based on the study conducted by Lenhardt *et al.* (2009), HSI value can be affected by several factors which are pollution, seasonal changes, type of nutrition available and number of population.

According to Chellapa *et al.* (1995), HSI analysis also can be used as estimation for energy level status in fish. The energy is in the form of lipid, protein, glycogen reserves in the liver. The energy stored in the liver is used for various living processes, including reproductive activity.

2.7 Fecundity

Fecundity is the measure of the rate of reproduction of any species, including *X. naritus*. In standard research methodology, fecundity is calculated by using egg production method, in which involving direct counting of total number of eggs in the ovaries of the female fish prior to spawning (Kingdom and Allison, 2011). This will give rise to the data of absolute fecundity. The data can be further related with other parameters, such as the length or weight of the individuals to determine the relative fecundity data. This is done in order to determine the reproductive potential of a female by measuring how many offspring they can produce at one time.

Besides, fecundity analysis is also done in order to assess the commercial potentialities of the fish stock in wild and to determine the abundance of the stock as well. Since *X. naritus* has become a regular food for local people in Betong, Sarawak, the fecundity analysis is a good way to study the population of this species in that area. The data can be used to monitor the fish stock in order to avoid overexploitation in future.

2.8 Maturity Stages

Generally, fish have their own life cycle. The stages in their life cycle may vary based on their species. In *X. naritus,* their life cycle and maturity stages are still not being documented. The closest attempt was done by Habib (1979) in order to describe pufferfish, *Uranostoma richei* from Lyttelton Harbor, New Zealand.

The determination of maturity stages is done by both naked eyes observation of the gonad condition and also histological analysis to confirm the cellular structures of the gonad. Then, the observation are described and placed under respective maturity stages by using the following seven-stage maturity scale Habib (1979) as reference.

In addition, the histological analysis is done by referring to previous study done by Yoshiharu *et al.* (1980), Sanchez et al. (2011) and Donald and Priscilla (1997) in order to correctly identify the microscopic structure of the gonad.

Table 1.Seven-stage maturity scale (Habib, 1979)

Maturity Stages	Description	
	Male	**Female**
I Immature	Testes a thin, translucent, spotted ribbon; no sperm	Ovaries thin, translucent, very small; eggs Microscopic
II Developing	Testes small, whitish; sperm a trace	Ovaries small, translucent to pink-tinged; eggs microscopic
III Maturing	Testes rounded, white; sperm more obvious	Ovaries rounded, pink to reddish; some eggs just visible by eye
IV Mature	Testes enlarged, opaque white; sperm very obvious, but not extruded with pressure	Ovaries enlarged and distended, reddish to orange; eggs clearly visible to eye, opaque
V Spawning	Testes very large, creamy white; full of sperm, some extruded with pressure	Ovaries very large and distended, reddish to orange; eggs large, some opaque, some transparent
VI Spent	Testes very large and distended, creamy white; sperm extruded with slight pressure; one testis may be partly flaccid and losing colour	Ovaries very large and distended, orange; eggs large, mature, transparent, extruded with slight pressure; one ovary may be partly flaccid and yellowish
VII Resting	Testes flaccid and shrinking, whitish to translucent; may still contain some sperm	Ovaries flaccid and shrinking, yellow; may still contain some eggs